APPENDIX J

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Differential effects of helper proteins encoded by the *cry2A* and *cry11A* operons on the formation of Cry2A inclusions in *Bacillus thuringiensis*

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Abstract

To compare the differential effects of *cry2A* operon *orf2* (29-kDa protein gene) and *Cry11A* operon *orf3* (20-kDa protein gene) on Cry2A synthesis and inclusion formation, we expressed the *cry2A* gene along with either the *29-kDa* gene, *20-kDa* gene, or both genes. Constructs containing *20-kDa*, in the presence or absence of *29-kDa*, produced more Cry2A than constructs which lacked this gene. Cry2A synthesis was also higher when the *29-kDa* gene was included with *20-kDa* in the construct. However, even in the presence of increased Cry2A synthesis facilitated by the *20-kDa* gene, typical Cry2A crystals did not form if the *29-kDa* gene was not included in the construct. These results suggest that the 29-kDa and 20-kDa proteins have different functions, with the 20-kDa protein acting like a molecular chaperone to enhance net Cry2A synthesis, and the 29-kDa protein likely serving as a template for the stabilization of Cry2A molecules and their organization into the rectangular inclusion characteristic of wild-type Cry2A crystals.

1. Introduction

One of the most interesting yet incompletely understood aspects of *Bacillus thuringiensis* is the genetic basis for the production of large crystals of Cry proteins. Typical 135-kDa Cry proteins such as Cry1Ac and Cry1C form bipyramidal crystals about 2 μm in length by 1 μm in width, making them as large or larger than the spores of most isolates [1]. It is thought that these large crystals result from separate genetic elements that promote, respectively, protein synthesis and crystallization. Evidence suggests that high levels of protein synthesis result from multiple and strong *cry* gene promoters, and very stable mRNAs [2–4], whereas crystallization is thought to be facilitated by the C-terminal half of Cry1 molecules and high levels of Cry1 synthesis during sporulation [5].

Smaller 65-kDa Cry proteins such as Cry2A, Cry3A, and Cry11A, lack the large C-terminus found in Cry1 proteins, but also form crystals in sporulating cells, though these are only a third to a fifth the size of Cry1 type crystals [1,6–8]. While

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there is evidence that promoter type, strength, and number as well as mRNA stability affect the levels at which these proteins accumulate in sporulating cells (2–4), other evidence suggests that helper proteins contribute to Cry2A and Cry11A net synthesis and crystallization. For example, *cry11A* occurs in an operon that encodes two other proteins, respectively, of 19 kDa and 20 kDa [9]. The 20-kDa protein, which occurs as the third open reading frame (ORF), enhances the synthesis of Cry11A [10], as well as other proteins [11,12], and facilitates the formation of larger Cry11A crystals [10]. The *cry2A* operon encodes a 29-kDa protein as the second ORF [6], and there is some evidence that it facilitates Cry2A crystallization [13].

Understanding the genetic basis for the high levels of Cry protein production and crystallization could provide insights into the biology of *B. thuringiensis* and contribute to the more effective use of Cry proteins in insect control. Toward this end, we studied the effects of the *cry11A* operon-encoded 20-kDa protein on the synthesis and crystallization of Cry2A in the presence and absence of the *cry2A* operon ORF2 protein. Here we show that including the *20-kDa* gene in *cry2A* constructs enhances net synthesis of Cry2A, but that typical Cry2A crystals only form when the *cry2A 29-kDa* gene is present in the constructs.

2. Material and methods

2.1. Plasmids and bacterial strains

The *cry2Aa1* operon, consisting of *orf1*, *orf2* and *orf3* (*cry2Aa1* gene, referred to herein as *cry2A*), was originally obtained from a 4.0-kb *Bam*HI-*Hin*dIII fragment in plasmid pCL-92 [14]. The *20-kDa* gene under control of the *cry1A(c)* promoters was cloned from pWF53 [10]. The various constructs of *cry2A* with and without the *cry2A orf2* and the *20-kDa* protein gene were made in the *Escherichia coli-Bacillus thuringiensis* shuttle vector pHT3101 [15] and amplified in *E. coli* strain DH5α. Recombinant constructs were expressed in *B. thuringiensis* strain 4Q7, an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis* (*Bacillus* Stock Center, Ohio State University, Columbus, OH, USA).

2.2. Construction of expression vectors

To express the intact *cry2A* operon in *B. thuringiensis* using pHT3101, the operon was first cut from the plasmid pCL-92 using flanking *Eco*RI and *Hin*dIII sites, cloned into the same sites in the polylinker of the phagemid Bluescript SK(±) (Stratagene, San Diego, CA, USA) for amplification and to add flanking restriction sites, and then cloned into the *Xba*I and *Sal*I sites of pHT3101. A similar strategy was used to generate a pHT3101-derived plasmid containing the *cry2A* operon minus *orf2*. The *orf2* gene was deleted from the *cry2A* operon by digesting pCL-92 with *Acc*I, thereby deleting two consecutive *Acc*I fragments bearing *orf2* and a portion of the 3′ end of *orf1*. Previous studies have shown that the product of *orf1* is not essential for Cry2A synthesis or crystallization [13]. Self-ligation of the resulting plasmid generated pPC2-1, from which the *cry2A* operon minus *orf2* was cut and cloned consecutively, as above, into pBluescript and then pHT3101. These constructions generated, respectively, the pHT3101 derivative plasmids pDBF42 (*cry2A* operon minus

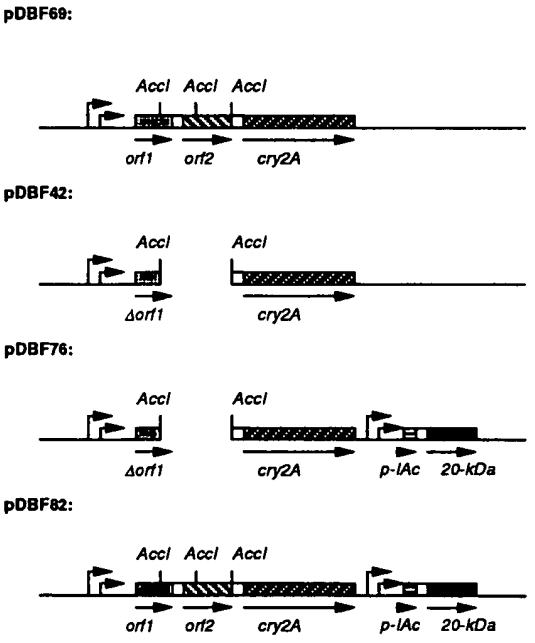

Fig. 1. Constructs of the *cry2A* operon used to determine the effects of the *cry11A* operon-encoded 20-kDa protein and Cry2A 29-kDa (*orf2*) protein on the production, crystallization, and formation of Cry2A inclusions.

orf2) and pDBF69 (*cry2A* operon) as shown in Fig. 1.

To determine the effect of the 20-kDa protein on the synthesis and crystallization of Cry2A in *B. thuringiensis*, a 1.3-kb *Sal*I-*Hin*dIII fragment containing the *20-kDa* gene under control of the *cry1Ac* promoters was cloned from pWF53 into pDBF42 and pDBF69 using the same sites. This generated, respectively, the pHT3101-derivative plasmids pDBF76 (*cry2A* operon minus *orf2* plus *20-kDa*) and pDBF821 (*cry2A* operon plus *20-kDa*) as shown in Fig. 1.

2.3. Bacterial transformation

Recombinant plasmids were transformed and amplified in *E. coli* strain DH5α according to standard procedures [16]. Plasmid DNA was purified using the Nucleobond AX system (The Nest Group, Inc., Southboro, MA, USA), and transformed into the *B. thuringiensis* strain 4Q7 by electroporation as described previously [17]. Transformants were grown and selected at 30°C on glucose-Tris (G-Tris) or Nutrient Agar containing erythromycin at 25 μg ml^{-1}.

2.4. Protein analysis and quantification

The amount of Cry2A produced with each construct was quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transformed *B. thuringiensis* 4Q7 strains were grown separately in 1 ml PWYE medium (5% peptone, 0.1% yeast extract, 0.5% NaCl, pH 7.5) overnight at 30°C, and then diluted 1:100 with 100 ml G-Tris medium containing 25 μg ml^{-1} erythromycin. Cultures were then grown at 37°C with shaking for 3–4 days, by which time more than 95% of the cells had sporulated and lysed. Spores, Cry2A crystals and cell debris were sedimented by centrifugation. The pellet was suspended in 50 μl Laemmli sample buffer and boiled for 5 min until completely dissolved. Protein content was determined by subjecting 50-μl samples to SDS-PAGE as described by Laemmli [18]. The gel was stained with 0.125% Coomassie Blue R-250, destained, dried, and the protein bands were scanned using a GAS 4000 Gel Documentation System (Evergene, Taiwan). The amount of protein in each band was quantified using ImageQuant 4.1 densitometry software (Molecular Dynamics, Sunnyvale, CA, USA). At least three different cultures were assayed for each of the constructs tested. Protein yields were analyzed statistically to determine mean levels of production and standard error about the mean.

2.5. Ultrastructural observations

Samples for electron microscopy were transferred to a 1.5-ml microfuge tube, pelleted by centrifugation, and fixed in 3% glutaraldehyde/0.25% sucrose for 2 h. Pellets were then post-fixed in OsO_4, dehydrated, and embedded in Epon-Araldite as described previously [17]. Ultrathin sections were cut on a Sorvall model MT5 microtome, stained with uranyl acetate and lead citrate, and examined and photographed with a Hitachi 600 electron microscope.

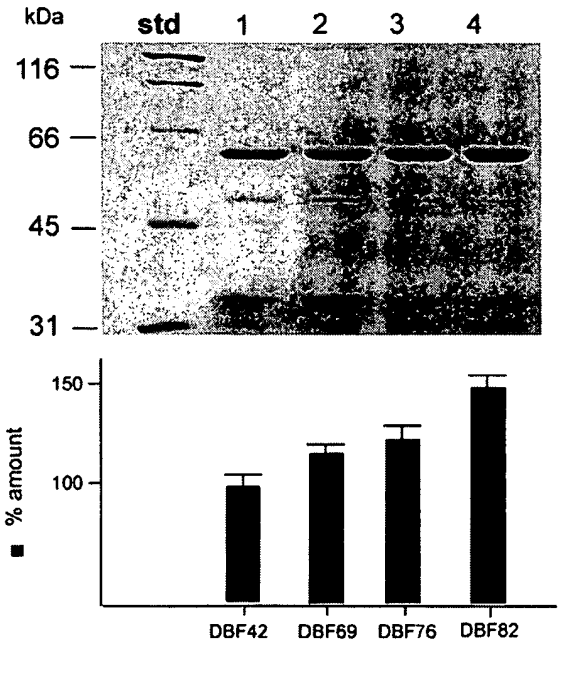

Fig. 2. Analysis of Cry2A yields produced by constructs of the *cry2A* operon with and with the *29-kDa* (*cry2A orf2*) and *20-kDa* (*cry11A orf3*) protein genes. Std, molecular mass marker proteins; Lane 1: *cry2A* operon minus *29-kDa*; lane 2: *cry2A* operon minus *29-kDa* plus the *20-kDa*; lane 3: *cry2A* operon; lane 4; *cry2A* operon plus *20-kDa* protein gene.

3. Results

3.1. Effects of the 20-kDa and 29-kDa proteins on Cry2A yield

The yield of Cry2A obtained when the *20-kDa* protein gene was included in the construct (pDBF76) was higher than when it was absent (pDBF42), and higher than that obtained with the *cry2A* operon (pDBF69) as illustrated in Fig. 2. Similarly, the presence of the *cry2A 29-kDa* protein gene in the constructs enhanced the yield of Cry2A (Fig. 2). More specifically, the *cry2A* operon (pDBF69) produced more Cry2A protein than the construct from which the 29-kDa gene (*orf2*) had been deleted (pDBF42). And the construct containing the *cry2A* operon and the 20-kDa (pDBF82) produced more Cry2A than the corresponding construct, pDBF42, which lacked the *cry2A orf2* gene (Fig. 2). Nevertheless, the highest yields were obtained when the construct contained both the *cry2A* operon and the 20-kDa protein gene (pDBF82). This construct produced approximately 1.25-fold the amount of Cry2A produced by the *cry2A* operon, and 1.5-fold the amount produced by the *cry2A* operon lacking *orf2* (Fig. 2).

3.2. Effects of cry2A 29-kDa and the 20-kDa proteins on Cry2A inclusion formation

In fully sporulated cells observed under phase microscopy, Cry2A inclusions were seen in all constructs which included the *29-kDa* gene (*orf2*). No Cry2A inclusions, however, were observed in cells transformed with constructs which lacked this gene.

To determine whether Cry2A formed inclusions smaller than those which could be readily observed with light microscopy, lysed cells were sedimented by centrifugation and the resulting pellet was subjected to SDS-PAGE analysis. No Cry2A protein was detected in the supernatant. Cry2A protein was only detected in the pellet of cell debris, indicating that Cry2A protein was insoluble in the absence of the 29-kDa protein, and probably formed small inclusions, but did not assemble into typical Cry2A crystals.

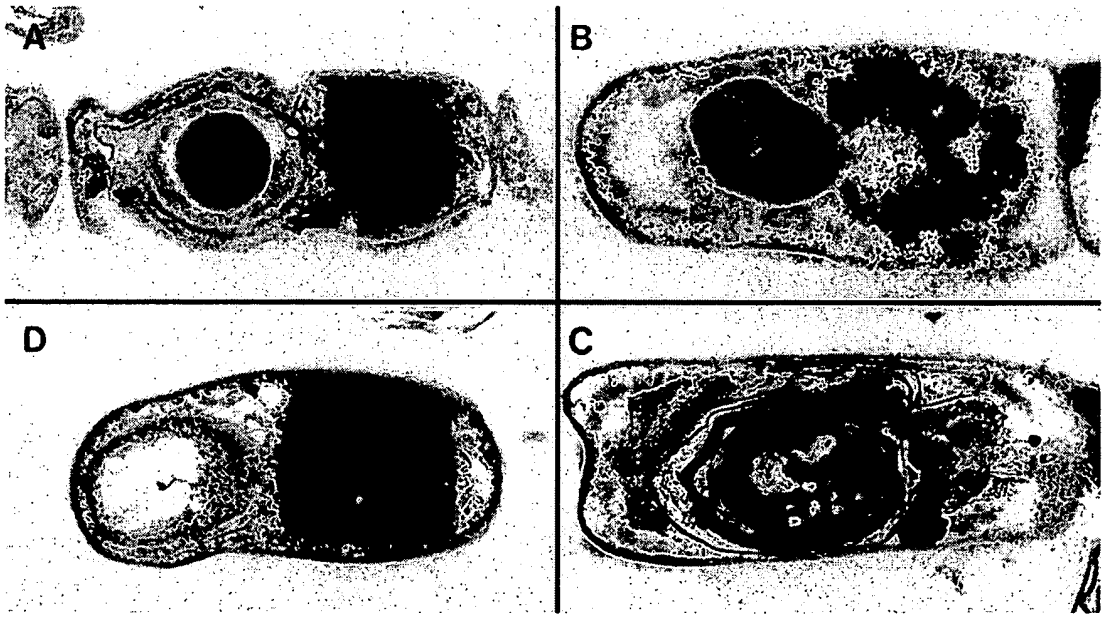

Fig. 3. Ultrastructure of typical sporulated cells of *Bacillus thuringiensis* obtained by expressing constructs of the *cry2A* operon with and without the *cry2A 29-kDa* gene and the *cry11A 20-kDa* gene. A: *cry2A* operon; B: *cry2A* operon minus *29-kDa*; C: *cry2A* operon minus *29-kDa* plus the *20-kDa* gene; D: *cry2A* operon plus the *20-kDa* gene. Bar in C equals 500 nm; all micrographs are the same magnification.

Evidence from transmission electron microscopy of sporulated cells just prior to lysis corroborated the results obtained with the constructs by SDS-PAGE analysis and light microscopy. Cells expressing the construct that contained the *cry2A* operon plus the *20-kDa* gene (pDBF82) typically produced larger Cry2A inclusions than the corresponding construct (pDBF76) that lacked this gene (Fig. 3). In cells which expressed the constructs lacking *cry2A orf2* gene encoding the 29-kDa protein (pDBF42, pDBF76), amorphous aggregates were observed, but no typical Cry2A inclusions (Fig. 3). A distinct lattice was observed in the aggregates formed in cells which expressed the pDBF42 construct, indicating that Cry2A could crystallize, but not form typical Cry2A inclusions in the absence of the 29-kDa protein (Fig. 4). No typical Cry2A inclusions were observed in cells expressing the *cry2A* operon which lacked the *orf2* gene (pDBF76) but contained the *20-kDa* gene (Fig. 3), suggesting that the 20-kDa protein could not replace the function of the 29-kDa protein.

Fig. 4. Higher magnification of the aggregates in *Bacillus thuringiensis* cells formed by the *cry2A* construct lacking the *29-kDa* (*orf2*) gene. Note that the aggregates do not form a typical Cry2A inclusion, but that a crystalline lattice is apparent in the aggregates. Bar equals 100 nm.

Though expression of the *cry2A* operon with or without the 20-kDa protein gene resulted in the formation of cuboidal crystals, we were unable to observe a lattice in these. This suggests that the lattice formed when the *orf2* gene is present in *cry2A* constructs may be different than that formed in its absence.

4. Discussion

Our results show that both the *cry2A* operon 29-kDa and *cry11A* operon 20-kDa proteins enhance Cry2A yields, but suggest that the mechanisms underlying these increases differ between the two proteins. While we obtained evidence that the 29-kDa protein enhanced Cry2A yield (Fig. 3), possibly by favoring crystallization of each molecule soon after synthesis, our ultrastructural studies suggest that its primary role is as a scaffolding protein, facilitating the crystallization of Cry2A molecules to form the cuboidal inclusion characteristic of this protein [13,14]. Alternatively, as has been shown with Cry11A [10] and Cry4A [11], the 20-kDa protein appeared to act like a chaperonin, enhancing the net yield of Cry2A synthesized, but not the formation of typical Cry2A inclusions (Fig. 3).

In principle, the information needed to specify the tertiary structure of a protein is contained in its primary amino acid sequence [19]. However, the primary sequence is often insufficient to direct the transition of a nascent monomeric polypeptide to the correct tertiary conformation. This typically requires the functional cooperation of a series of molecular chaperones to obtain proper protein folding [20]. In *B. thuringiensis*, it is thought that the conserved C-terminal half of the typical 135-kDa Cry proteins such as Cry1A, Cry4A and Cry4B serves to stabilize the crystals that these molecules form [3,21,22]. This is supported by the observation that intermolecular disulfide bridges form between cysteine residues of the C-terminus [5]. Smaller proteins like Cry11A and Cry2A, however, lack the corresponding C-terminus [1] and associated disulfide bridges, and therefore appear to require additional

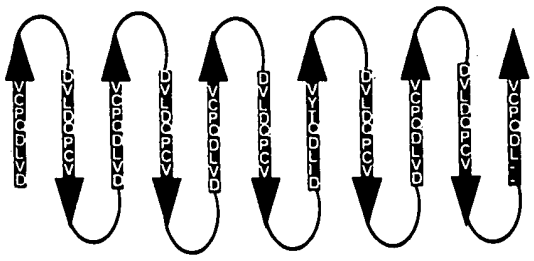

Fig. 5. Theoretical template that could serve as a nucleation site for Cry2A molecules. The template is formed by the tandem repeats of a 15-amino acid sequence in the *cry2A orf2* 29-kDa protein, and is based on computer simulations [24] and on similarities to ice nucleation proteins [25].

proteins to optimize stabilization and crystallization. Thus, the apparent role of the *cry11A* 20-kDa and *cry2A* 29-kDa proteins, respectively, is to enhance the production and crystallization of the Cry proteins, especially of those with which they occur naturally.

With respect to the 20-kDa protein, our finding that a higher yield of Cry2A yield was obtained when the gene for this protein was included in the construct, with or without *cry2A 29-kDa* gene (Figs. 2 and 3) was likely due to the stabilization of the newly synthesized Cry2A molecules. This 20-kDa protein could have achieved this by protecting the Cry2A molecules from proteolysis and by assisting the efficient protein folding. In support of this hypothesis, it has been shown that the requirement for 20-kDa protein in Cyt1A synthesis can be bypassed to some extent in *E. coli* strains that produce heat shock chaperone proteins that retard proteolysis [22,23]. Nevertheless, enhancement of Cry2A synthesis by the 20-kDa protein did not lead to typical Cry2A inclusions, suggesting that 20-kDa protein could not provide the scaffolding function of the 29-kDa protein.

In the case of the 29-kDa protein, crystalline protein aggregates, but not typical Cry2A inclusions were obtained in all constructs which lacked the *29-kDa* gene (Fig. 3). The transition of Cry proteins into an ordered state by crystallization is thought to stabilize the proteins by protecting them from proteolysis [3]. Thus, increased production of Cry2A might be enhanced by its more rapid and orderly crystallization in the presence of the 29-kDa protein. A more definitive role for this protein remains to be determined, but its secondary structure suggests it is a scaffolding protein that could function in two ways: to ensure proper folding of newly synthesized molecules, and to prevent their aberrant aggregation, favoring crystallization to form typical Cry2A inclusions. These roles receive some support from an analysis of Cry2A structure. For example, the deduced amino acid sequence of the 29-kDa protein shows a sequence of 15 amino acids repeated almost perfectly in tandem eleven times [6]. Secondary structure predictions [24] for this protein reveal these repeats could form a set of ordered β-sheets (Fig. 5) which, either separately or when 29-kDa molecules are grouped together, could form a scaffold facilitating proper folding and crystallization of Cry2A molecules. Such a scaffold would not be necessary for crystallization, but could be required for the formation of characteristic Cry2A crystals. Precedent for this is found in the ice-nucleating protein of *Pseudomonas fluorescens* [25]. This protein, which also contains tandem amino acid repeats, occurs in the outer bacterial membrane and provides nucleating sites for water leading to the formation of ice at temperatures above 0°C [25].

Finally, although 20-kDa and 29-kDa proteins were each found to increase the synthesis of Cry2A, the maximum yield was obtained when both of their encoding genes were included in the same construct. These results indicate the two helper proteins identified to date can function cooperatively to assure the efficient production of Cry2A in *B. thuringiensis*, apparently by affecting the different aspects of the protein production process. If so, there might exist sequential interaction of the 20-kDa protein and the 29-kDa protein with target Cry2A protein, which could be not only of basic interest but of practical application for producing higher yields of Cry2A.

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References

[1] Hofte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242–255.

[2] Agaisse, H. and Lereclus, D. (1995) How does *Bacillus thuringiensis* produce so much insecticidal crystal protein. J. Bacteriol. 177, 6027–6032.

[3] Baum, J.A. and Malvar, T. (1995) Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Mol. Microbiol. 18, 1–12.

[4] Glatron, M.F. and Rapoport, G. (1972) Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. Biochimie 54, 1291–1301.

[5] Bietlot, H.P.L., Vishnubhatla, I., Carey, P.R., Pozsgay, M. and Kaplan, H. (1990) Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem. J. 267, 309–315.

[6] Widner W.R. and Whiteley, H.R. (1989) Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* susp. *kurstaki* possess different host range specificities. J. Bacteriol. 171, 865–974.

[7] Donovan, W.P., Dankocsik, C.C., Gilbert, M.P., Gawron-Burke, M.C., Groat, R.G. and Carlton, B.C. (1988) Amino acid sequence and entomocidal activity of the P2 crystal protein. J. Biol. Chem. 263, 561–567.

[8] Sekar, V., Thompson, D.V., Maroney, M.J., Bookland, R.G. and Adang, M.J. (1987) Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. USA 84, 7036–7040.

[9] Dervyn, E., Poncet, S., Klier, A. and Rapoport, G. (1995) Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 177, 2283–2291.

[10] Wu, D. and Federici, B.A. (1995) Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using *cryIA(c)* promoters to express the gene for an associated 20-kDa protein. Appl. Microbiol. Biotechnol. 42, 697–702.

[11] Yoshisue, H., Yoshida, K., Sen, K., Sakai, H. and Komano, T. (1992) Effects of *Bacillus thuringiensis* var. *israelensis* 20-kDa protein on production of the Bti 130-kDa crystal protein in *Escherichia coli*. Biophys. Biotechnol. Biochem. 56, 1429–1433.

[12] Rang, C., Bes, M., Lullien-Pellerin, V., Wu, D., Federici, B.A. and Frutos, R. (1996) Influence of the 20-kDa protein from *Bacillus thuringiensis* ssp. *israelensis* on the rate of production of truncated CryIC proteins. FEMS Microbiol. Lett. 141, 261–264.

[13] Crickmore, N. and Ellar, D. (1992) Involvement of a possible chaperonin in the efficient expression of a cloned CryIIA delta-endotoxin gene in *Bacillus thuringiensis*. Mol. Microbiol. 6, 1533–1537.

[14] Moar, W.J., Trumble, J.T., Hice, R.H. and Backman, P.A. (1994) Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *E. coli* and *B. thuringiensis* and in a leaf colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. 60, 896–902.

[15] Lereclus, D., Arantes, O., Chaufaux, J. and Lecadet, M.M. (1989) Transformation and expression of a cloned δ-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. 60, 211–218.

[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[17] Wu, D. and Federici, B.A. (1993) A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. 175, 5276–5280.

[18] Laemmli, U.K. (1970) Cleavage of structural proteins during the assemply of the head of bacteriophage T4. Nature 227, 680–685.

[19] Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. Science 181, 223–230.

[20] Hendrick, J.P. and Hartl, F.U. (1993) Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. 62, 349–84.

[21] Aronson, A.I. (1993) The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. Mol. Microbiol. 7, 489–496.

[22] Adams, L.F., Visick, J.E. and Whiteley, H.R. (1989) A 20-Kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J. Bacteriol. 171, 521–530.

[23] Visick, J.E. and Whiteley, H.R. (1991) Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on the production of the CytA protein by *Escherchia coli*. J. Bacteriol. 173, 1748–1756.

[24] Garnier, J., Gibrat, J.F. and Robson, B. (1996) GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol. 266, 540–553.

[25] Kajava, A. and Lindow, S.E. (1993) A model of the three-dimensional structure of ice nucleation proteins. J. Mol. Biol. 232, 709–717.